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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 13/00, A61K 35/14
C07H 21/00

A1

(11) International Publication Number: WO 91/16350

(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/02607

(22) International Filing Date: 16 April 1991 (16.04.91)

(30) Priority data:
512,132 20 April 1990 (20.04.90) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: ABERRANT, EPIDERMAL GROWTH FACTOR RECEPTOR DNA, RNA AND PROTEIN FORMS AND METHOD

(57) Abstract

The aberrant genomic codes for epidermal growth factor receptor (EGFR) are found in tumor tissue especially glioblastomas. These can be co-expressed with normal EGFR and are amplified. These can serve as tumor markers, used to assay effects of therapy or as therapy guidelines and to classify tumor types. These tumor types can then serve as further therapy guidelines. Aberrant EGFR protein can be used to produce monoclonal antibody for diagnostic purposes or for therapy if combined with a label or a toxin.

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ABERRANT, EPIDERMAL GROWTH FACTOR RECEPTOR DNA, RNA AND PROTEIN FORMS AND METHOD

This invention concerns aberrant DNA, RNA and protein forms of epidermal growth factor receptor. These forms can be used as tumor markers, used to assay effects of therapy or as therapy guidelines and to classify tumor types. These tumor types can then be used for further therapy guidelines.

Description of the Drawing

Fig. 1 a) EGFR cDNA (3816bp coding sequence) is described with respect to the regions coding for structural/functional domains of corresponding protein[26]. SP:signal peptide TM:transmembrane.

b) Enlargement of the first 1500 bases showing positions and sizes of the exons and introns 1 through 7. Shaded region: coding sequences eliminated from aberrant transcripts in the six glioblastomas examined. Locations of oligonucleotides used in the study are indicated. Numerical sequence identities of oligonucleotides (5′ to 3′) based upon Ullrich et al. 1984[26], PC46=50-1; PC56=1-50; PC66=172-193; PC15=428-379; PC29=914-865; PC83=979-1000; PC84=1000-1021; PC88=1054-1075; PC89=1097-1076; PC85=1099-1078; PC77=1167-1146; PC59=1250-1201; PC67=1356-1335; PC58=1490-1441. In addition a cDNA probe, pE7 (including approximately bases 650-3000[14]), was used on SacI digested DNA for screening for aberrant restriction fragments (RF) in the tumors. Oligonucleotides used to examine amplified genes 3′ of the deleted region (not shown; all anti-sense): PC54=1850-1801; PC17=2100-2051; PC64=2549-2500; PC63=2899-2850; PC47=3299-3250; PC34=3699-3650.

Fig. 2. Genomic analysis of EGFR rearrangements. Tumor (T) DNA from three patients, P_1-P_3 were hybridized with pE7 and PC59 (see Fig. 1). A normal SacI restriction fragment (RF) pattern resulting from hybridization of pE7 is displayed for P_1. Hybridization of pE7 to each of the tumor DNAs revealed a relative depletion of the normal 10kb SacI

RF. The 1.75kb RF identified by pE7 in normal DNA is also identified by PC59. For tumor DNAs from P_1 and P_2, hybridization with PC59 revealed additional, tumor specific, RF's of 2.4kb (P_1) and 6.5kb (P_2). These aberrant bands were also revealed by pE7 hybridization, although the aberrant 6.5 kb RF of P_2 was somewhat obscured by a normal RF of similar size. These aberrant RF's result from the deletion rearrangement elimination of the SacI site defining the 5' and of 1.75kb fragment, located in intron 7. Patient P_3 showed no rearrangement of the 1.75kb fragment. However, use of an intron 7 probe, Int7 (consists of 22 bases of exon 7, all of intron 7, and 24 bases of exon 8) revealed, in addition to the normal 1.75kb and 3kb RF, an aberrant 5kb RF indicating that the 3' end of the rearrangement in this tumor occurred 5' of the SacI site in intron 7.
METHODS: DNA isolation, digestion, Southern blotting and radiolabelling of pE7 and Int7 by random oligonucleotide priming, as previously described [21,27]. Radiolabelling of oligonucleotide probes by 3'extension (terminal transferase, $^{\alpha-32}$PdCTP). Int7 produced by PCR exon-exon connection strategy with oligoprimers PC84 and PC85 from normal DNA.

Fig. 3 a) An autoradiogram composite indicating aberrant EGFR transcripts in glioblastomas. Upper part constructed by surprimposing horizontally displaced autoradiograms (open lane between samples) resulting from hybridization of PC29 and rehybridization of PC59 to the same filter. The lower portion represents an autoradiogram resulting from rehybridization of a GAPDH oligonucleotide probe (50bp) to the same filter, to check sample quantity. A431 cells which have amplified EGFR and show a 10kb and a 2.9kb transcript [19] were included as a control. P_4 shows expression of a normal 10kb transcript when probed with PC29 (only visible at long exposures; arrow) as well as an aberrant, overexpressed, shortened transcript which is detected with PC59. P_1 shows an overexpressed 10 kb transcript as well as the same phenomenon as P_4.

METHODS: Total RNA isolated from frozen tumor tissue by polytrone homogenization in guanidine isothiocyanate buffer followed by ultracentrifugation on a CsCl gradient. Twenty μg RNA was electrophoresed on a denaturing 1% agarose gel, blotted to Hybond N^R membrane and hybridized to radiolabelled synthetic oligonucleotides (see Fig. 2).

b) Ethidium bromide stained gel of the products from PCR amplification of EGFR cDNA with primer pair PC66 and PC67 (Fig. 1). Lanes 1 and 10, 123bp ladder; lanes 2 and 3, product from two specimens of non-neoplastic brain, removed in the surgical treatment of epilepsy; lanes 4-9, products from the six tumors P_1-P_6.

METHODS: Total RNA isolated as in Fig. 3a. Single stranded cDNA produced using MoMuLV reverse transcriptase and random priming with hexanucleotides [28]. PCR reaction: 30 cycles; 94°C/1 min.; 55°C/1 min.; 72°C/3 min. + 10 sec/cycle, last cycle 10 min.

c) Southern blot of b) probed with PC59 (see Fig. 1). In autoradiograms exposed longer the normal 1185bp band could be shown in all samples.

d) Southern blot of b) probed with PC29 (see Fig. 1). Hybridizing with PC15 (not shown) gave similar results (in autoradiograms exposed longer the 1185bp band could be shown in all samples).

METHODS: c) and d) alkali blotting to Gene-Screen-$Plus^R$ nylon membranes, oligonucleotides radiolabelled as for Fig. 2.

Fig. 4. Autoradiogram of a sequence gel (reading anti-sense) from P_6 (left) and P_1 (right). A comparison of this sequence with that reported [24, 25] for the first exon of EGFR shows that the last 70 nucleotides (only 26 shown) before the 5′ splice site, in the sense sequence are identical to the last 70 nucleotides in exon 1. The aberrant splicing (splice point arrowed) occurs after the first base in a condon (last base in exon 1) and continues with the last two bases from another condon (first two bases

of xon B, see text), thus th rearranged mRNA remains in-frame with the loss of 267 condons and the production of a new codon "GGT" (Gly). The aberrant mRNA codes for the normal signal peptide and a truncated EGFR consisting of the first five N-terminal amino-acids, the loss of the following 267 amino-acids, the addition of a glycine, and then remains in-frame reading from condon 274.

METHODS: Double stranded cDNA was produced and amplified as described in Fig. 3 and isolated from a 1% agarose gel using Gene-Clean^{R}. cDNA (1.5 ng) was then used in a unbalanced PCR reaction (30 cycles; 94°C/1 min.; 55°C/1-min.; 72°C/3 min. + 10 sec./cycle, last cycle, 10 min. Primers: PC66, 50pM; PC67, 1.5pM) to produce a sense single stranded (ss) cDNA template which was isolated from a 1% agarose gel by freeze-thawing. Approximately 100ng of this sscDNA was then primed with PC77 (0.6pM) and chain termination sequenced using a Sequence^{R} kit (U.S. Biochem. Corp.) according to the manufacturers recommendations.

Description of the Invention

Several molecular anomalies have been documented in glioblastoma and maybe responsible for their malignant phenotype. Amplification, overexpression and rearrangements of EGFR have been reported in glioblastomas and studies of glioma cell lines have suggested the possibility of auto- or paracine loops involving TGF α/EGFR in these tumors. We have studied gene dose, gene structure as well as the structure and level of mRNA transcripts for EGFR, TGFα and EGF in a series of 30 primary human glioblastomas. DNA and RNA was studied on Southern and Northern blots respectively using anti-sense synthetic oligonucleotides to different parts of the EGFR, EGF and TGFα genes. For detailed studies, dscDNA was randomly produced from total RNA by reverse transcription followed by specific PCR amplification. Unbalanced PCR was used to produce sscDNA templates for dideoxy sequencing.

The gene for EGFR was amplified between 9-112 times

in just ver 50% of the tumors. Structural abnormalities in the amplicon were detected in 43% of the amplified cases. Five of six aberrantly amplified cases showed truncated transcripts on Northern blots. Aberrant transcripts were also found in cases with apparently normal restriction fragments of the gene as well as in tumors with non-amplified normal genes. The structural abnormalities clustered in either the extracellular or intracellular domains. PCR amplification and nucleotide sequencing of tumor mRNA revealed an identical loss of sequence affecting the extracellular domain in 5 studied cases. In addition, we found expression of mRNA for EGF at low but detectable levels and/or TGFα at varying levels in all glioblastomas studied.

Glioblastomas may have an amplified, overexpressed, and in a large percentage of cases a structurally altered EGFR. They also express either EGF or TGFa or both. The aberrations of EGFR are commonly found in the extracellular region and are identical in many cases. The truncated receptor may function analogously to the erb-B gene product.

The epidermal growth factor receptor (EGFR) gene has been found to be amplified and rearranged in human glioblastomas <u>in vivo</u> [1-9]. However, no sequence details of such rearrangements have been published. Here we present the sequence across a novel splice junction of aberrant EGFR transcripts, derived from corresponding and uniquely rearranged genes, which are co-amplied and co-expressed with non-rearranged EGFR genes in six, primary, human glioblastomas. Each of the six cases examined reveals aberrant transcripts derived from identical splicing of exon 1 to exon 8 as a consequence of a deletion-rearrangement of the amplified gene, the extent of which is variable amongst these tumors. In spite of their intertumoral variability, these intragenic rearrangements result in the loss of 801 coding bases (exons 2 through 7) and creation of a new codon at the novel splice site in their corresponding transcripts.

The mRNA sequence for the signal peptide, the first five codons, and the reading frame 3′ of the rearrangement, are intact.

The EGFR protein is a 170kD transmembrane glycoprotein found on many normal and malignant cells [1, 10-15]. The extracellular binding of one of its two known endogenous ligands, epidermal growth factor and transforming growth factor α, results in conformational changes of the extracellular domain [16], the activation of the receptor's intracellular tyrosine kinase activity [17, 18], and the stimulation of DNA synthesis. A constitutively activated, and cell transforming variant of this receptor, with most of the extracellular domain deleted and further carboxyterminal deletions and mutations, is encoded by the v-erb B oncogene of avian erythroblastosis virus [19,20].

EGFR gene amplification is exclusively seen in the most malignant variants of gliomas, especially the glioblastomas [21]. Studies of DNA, mRNA, and protein from primary human glioblastomas and xenografted glioblastomas with EGFR amplification, indicate the existence of truncated EGF receptors in such tumors [1-9]. Most results implicate the loss of coding sequences for the extracellular domain [5,6,8].

We have studied the DNA and RNA from primary tumor tissue from six patients with brain tumors histopathologically classified as glioblastomas [22, 23] where the tumors were determined to have amplification of the EGFR gene [21]. Using a series of EGFR mRNA complementary oligonucleotide probes (see Fig. 1) on Southern blots, it was possible to determine a region of the gene which, amongst these tumors, displayed a relatively reduced level of amplification (sequences complementary to PC15 and PC29; see, for example, the 10kb (recognized by PC15) restriction fragment (RF) in the tumor DNA of patient P_1, see Fig. 2). According to Haley et al. [24], this region lies between intron 1 and intron 7. Use of the oligoprobe PC59, whose sequence

corresponds to the 3′ end of exon 8 (Fig. 1), revealed an amplified, tumor DNA specific, rearranged SacI RF in some of the cases (see P_1, P_2 in Fig. 2). Intertumoral variable amounts (Fig. 2) of an amplified normal 1.75kb FR (SacI sites in introns 7 and 8) were present in addition to the aberrant RFs, and the ratio of the amplified rearranged RF was unique to each tumor. In contrast some patients, including patient P_3 (Fig. 2) showed no rearrangement of the 1.75kb SacI RF. However, for patient P_3, a rearrangement of the contiguous 5′ normal 3kb RF, identified by Int7 [see Fig. 2 for details] and PC29, was identified. These data demonstrate the variability in genomic location of the 3′ end of the intragenic deletion-rearrangement. Since the variation in the sizes of the aberrant fragments detected by PC59 in P_1 and P_2 (4.1kb) cannot be accounted for by differences in the position of the rearrangement site in the 1.75kb normal fragment, these data indicate variability of the 5′ end of the rearrangement as well.

The Northern blot analysis showed variable expression of the normal 10kb transcript in all cases. In addition, overexpressed aberrant transcripts could be detected in at least five of the cases, two examples of which are shown in Fig. 3a. The aberrant transcripts hybridized to all antisense oligonucleotides (Fig. 1) except PC29 (Fig. 3a) and PC15 (not shown). As will be shown below, the aberrant transcripts lacked an internal stretch of 801 bases.

To determine the consequences of these rearrangements on the EGFR mRNA, a pair of oligonucleotide primers containing sense sequences 5′ (exon 1) and antisense sequences 3′ (exon 8) of the deleted region were used for the PCR amplification of EGFR cDNA from each tumor. This procedure should normally result in the amplification of a 1185bp DNA fragment (bases 172-1356 of mRNA). However, in all cases an abnormal fragment of appox. 380bp, in addition to the normal 1185bp fragment were observed (Fig. 3c). The

yield of the latter band varied between tumors. As anticipated, oligoprobes PC15 and PC29 hybridized to the normal fragment, but not to the shorter, aberrant fragment (Fig. 3d) whereas PC59 detected both fragments (Fig. 3c). We thus concluded that this 380 bp fragment includes the flanking coding sequences on either side of the deletion, and that all the tumors appeared to have lost approximately the same number of bases from the aberrant EGFR transcript. Sequencing of the this 380bp fragment (Fig. 4) revealed that the last nucleotide of the first exon (base 274) [24, 25] had been spliced to base 1076 in each of the six tumors. This novel splice results in the creation of a glycine codon (GGT) at the sixth codon position and the in-frame apposition of what would normally be codon 273 to the seventh codon position of the aberrant transcript.

Our results indicated that these aberrant transcripts contain the entire exon 1 [24, 25] followed by a sequence starting at base 52 of the 150 bases long exon 8 [24]. To clarify this further, the position and size of intron 7 was determined by using an exon-exon connection strategy. A contiguous primer pair, one ending at sence base 1075 (hypothesized as being the last nucleotide in exon 7) (PC88) and the second ending at anti-sense-base 1076 (hypothesized as being the first nucleotide in exon 8) (PC89), was used to PCR amplify non-coding DNA which might interrupt these sequences. The PCR product using these primers on two different normal DNA templates (white blood cells) was approximately 1780bp (data not shown). This result is consistent with the hypothesis that intron 7 lists between bases 1075 and 1076 and thus the reported 3′ and 5′ ends of introns 7 and 8 respectively [24] are incorrect. To support the EGFR-gene authenticity of this fragment, 5′ sense and 3′antisense primers, outside PC88 and PC89, were substituted for one or the other of the original primers to amplify a slightly larger genomic fragment containing known coding sequences (approx. 1850bp). As expected, the primers

internal to the primers used, hybridized to the amplified product in each case. On the basis of these data, we conclude that the sequence observed in the aberrant mRNA transcripts results from the splicing of exon 1 to exon 8. This would explain how the internal deletion in the mRNA transcript is always the same, despite the fact that the sites of the genomic deletion-rearrangement varies in individual tumors (Fig. 2).

It is noteworthy that the aberrant EGFR gene/transcript has only been found in tumors also having a non-rearranged amplified gene and therefore would presumably arise during the amplification process; in 66 gliomas we have studied, no gross genomic or transcript rearrangement has been observed in the absence of amplification (Ekstrand J. in preparation). Tumors with an amplified, rearranged gene co-express normal and aberrant transcripts. Similar rearrangements may also occur in the other tumor types which show amplification of the EGFR gene [11-13]. The finding of identical abnormal splicing of exon 1 to 8 in the primary glioblastomas of six different patients suggests that the corresponding truncated EGFR has biological significance. An identical deletion in the EGFR transcript has been found in two xenografts of glioblastomas.

The coding region lost is the N-terminal, cysteine rich domain, with possible involvement of the N-terminal portion of the ligand binding domain. The presence of the signal peptide with five N-terminal amino acids and in-frame coding sequence 3′ of the rearrangement should ensure proper sorting and membrane insertion, respectively, of the aberrant protein. It seems an unlikely coincidence that the 801 bases deleted from the transcripts reported here would account for the molecular weight differential observed between the normal 170kD receptor and the aberrant 140kD receptor reported in two xenografted glioblastomas [6]. Cell membrane preparations from the two xenografts containing the aberrant 140kD EGFR protein showed a significant elevation

of tyrosine kinase activity in the absence of ligand [6]. This suggests an aberrant EGFR functionally analogous to the constitutively kinase-active retroviral homologue of the EGFR protooncogene, v-erb B, the expression of which can transform, for example, fibroblasts [20].

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The examples herein are intended to illustrate the invention. They are not intended to limit the invention to the specific examples herein. Other examples will suggest themselves to those skilled in the art.

Claims

1. Aberrant EGFR cDNA.

2. Aberrant, rearranged EGFR cDNA capable of co-amplification and co-expression with non-rearranged cDNA in tumors.

3. The cDNA of claim 2 wherein the tumor is a glioblastoma.

4. The cDNA of claim 2 or 3 resulting in loss of 801 coding bases comprising exons 2-7 with a resulting new codon at a novel splice site wherein the EGFR cDNA for the mRNA sequence for the signal peptide, the first five codons and the reading frame 3′ of the rearrangement remain intact.

5. Oligonucleotide probes for normal and aberrant EGFR comprising at least one selected from the group consisting of Int7, PC46, PC56, PC66, PC15, PC29, PC83, PC84, PC88, PC89, PC85, PC77, PC59, PC67, PC58, PE7, PC54, PC17, PC64, PC63, PC47 and PC34.

6. Aberrant mRNA expressing protein coded for by the cDNA of claim 1, 2, 3 or 4.

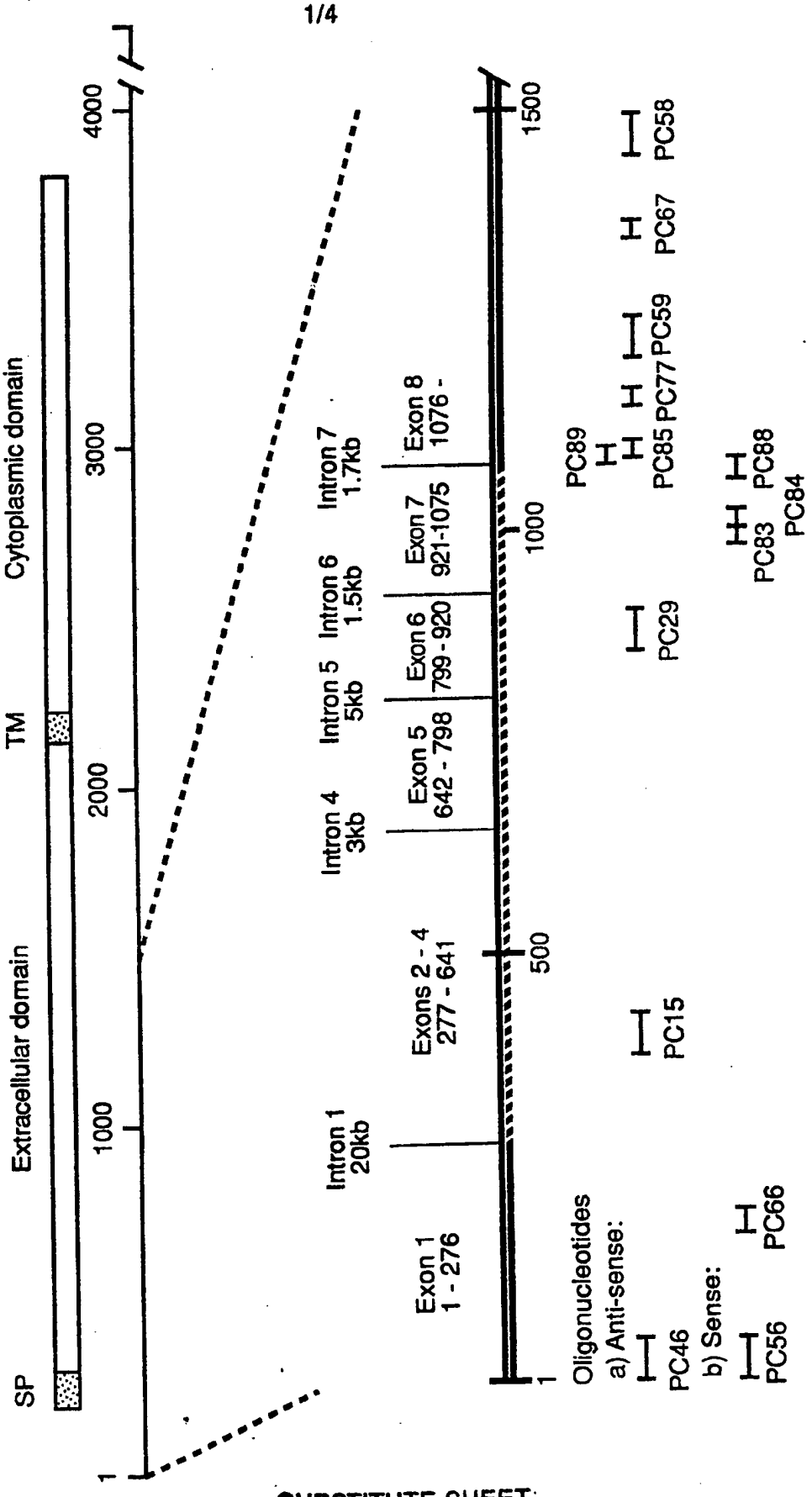
7. The aberrant, rearranged EGFR protein resulting from expression of the cDNA of claim 1, 2, 3 or 4.

8. The cDNA of claim 4 wherein the new splice site codon codes for glycine.

9. The cDNA of claim 2 or 3 expressing aberrant EGFR transcripts comprising exon 1 followed by a sequence starting at base 52 of the 150 base long exon 8.

10. Monoclonal antibody resulting from the protein antigen of claim 7.

FIG. 1.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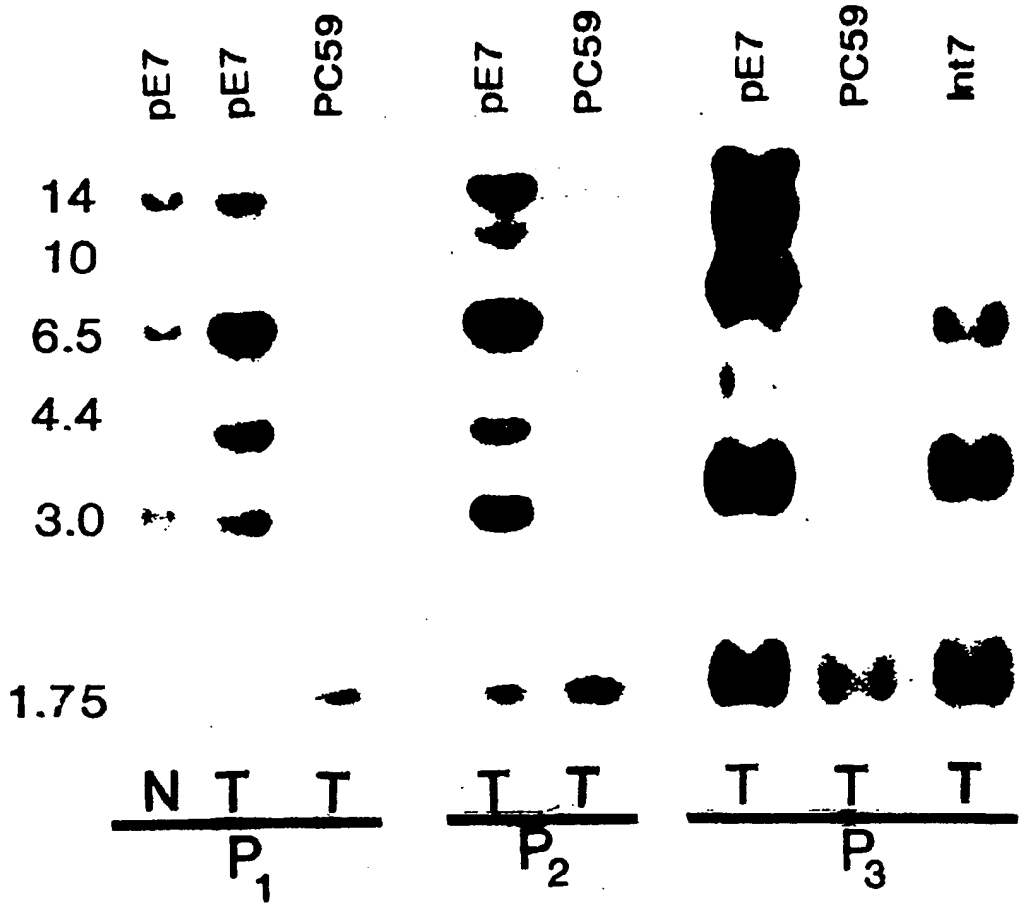
Extracellular domain
TM
Cytoplasmic domain
1
1000
2000
3000
4000
Exon 1
1 - 276
Intron 1
20kb
Exons 2 - 4
277 - 641
Intron 4
3kb
Exon 5
642 - 798
Intron 5
5kb
Exon 6
799 - 920
Intron 6
1.5kb
Exon 7
921-1075
Intron 7
1.7kb
Exon 8
1076 -
1
500
1000
1500
Oligonucleotides
a) Anti-sense:
PC46
PC15
PC29
PC89
PC85
PC77
PC59
PC67
PC58
b) Sense:
PC56
PC66
PC83
PC88
PC84

FIG. 2.


pE7
pE7
PC59
pE7
PC59
pE7
PC59
ht7
14
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4.4
3.0
1.75
N T T
T T
T T T
P1
P2
P3

FIG. 3.

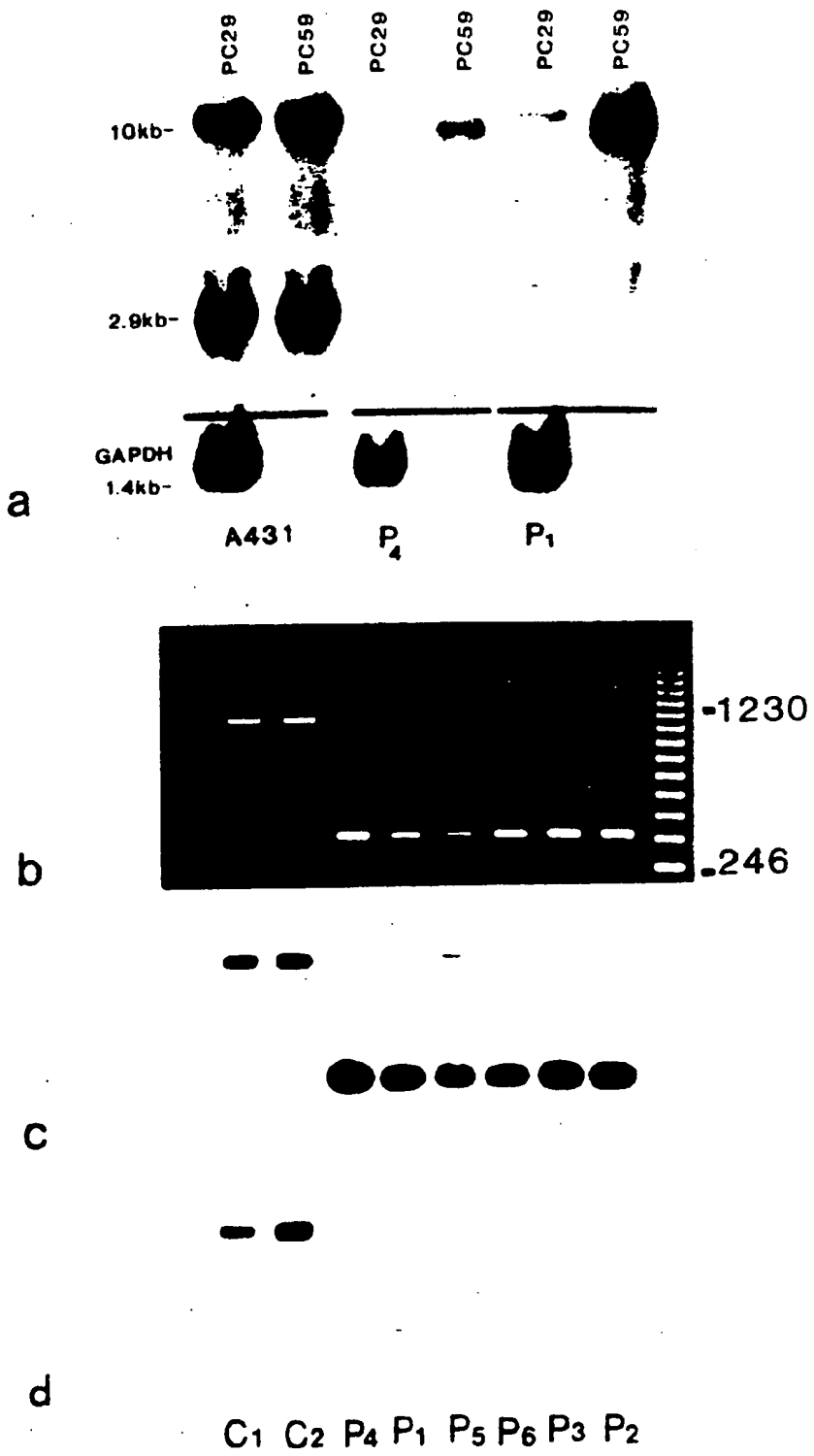
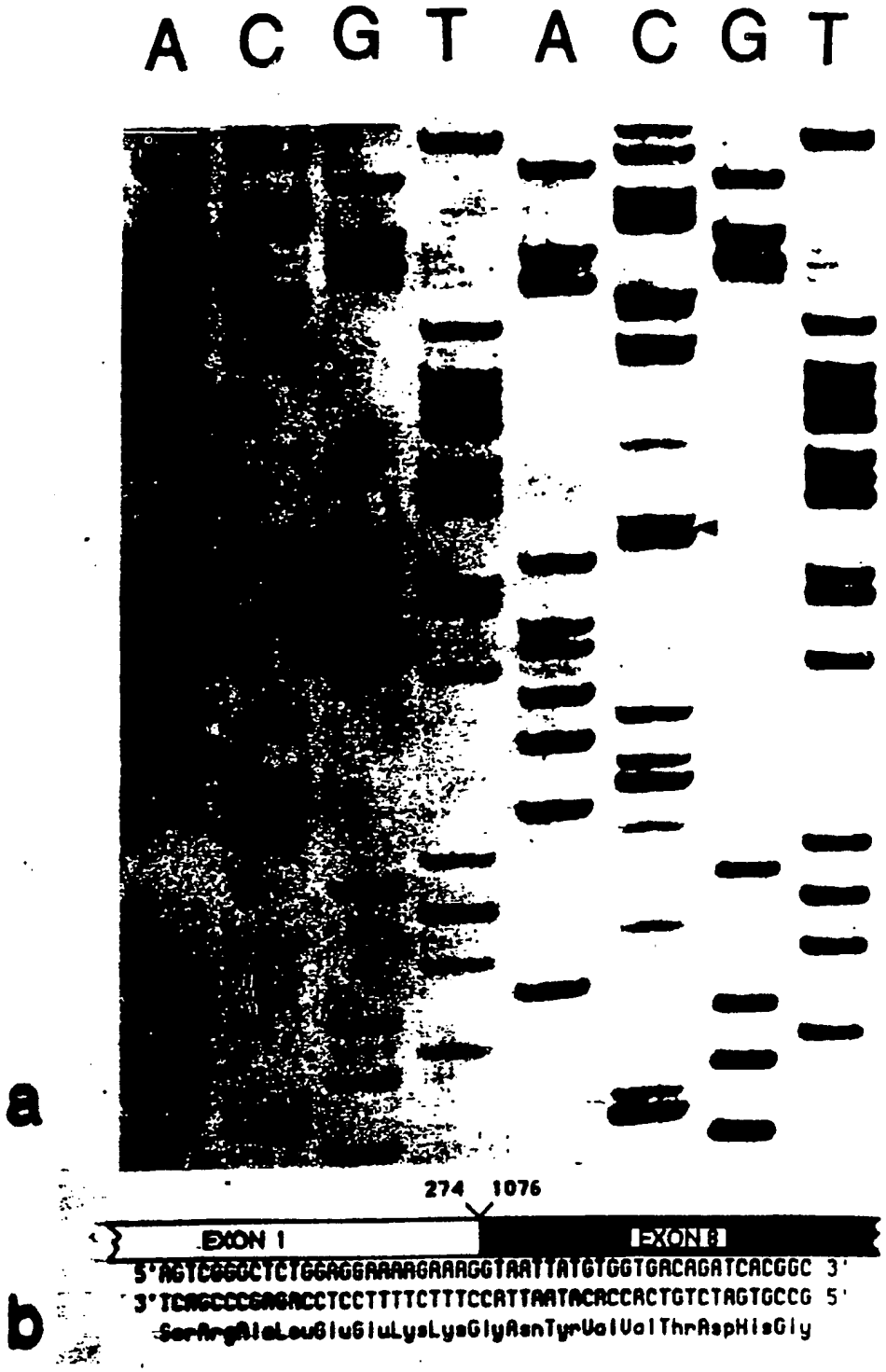

PC29
PC59
PC29
PC59
PC29
PC59
10kb-
2.9kb-
GAPDH
1.4kb-
a
A431
P4
P1
b
-1230
-246
c
d
C1 C2 P4 P1 P5 P6 P3 P2

FIG. 4.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274
1076
EXON 1
EXON II
5'AGTCGGCCTCTGGAGGAAAAGAAAGGTAATTATGTGGTGACAGATCACGGC 3'
3'TCAGCCCGGAGACCTCCTTTTCTTTCCATTAATACACCACTGTCTAGTGCCG 5'
SerArgAlaLeuGluGluLysLysGlyAsnTyrValValThrAspHisGly
b

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02607

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 530/350,387; 536/27
IPC(5): C07K 13/00; A61K 35/14; C07H 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	530/350,387; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS,APS, MEDLINE, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	JOURNAL OF CELLULAR BIOCHEMISTRY, SUP PL. 13B, ISSUED 1989, A.U. WONG ET AL. "INTERNAL DELETIONS OF THE EGF RECEPTOR GENE IN PRIMARY HUMAN GLIOMAS," PAGE 149, SEE ENTIRE ABSTRACT.	1-6
Y	MOLECULAR AND CELLULAR BIOLOGY, VOLUME 8, NO. 4., ISSUED APRIL 1988, H. YAMAZAKI ET AL," "AMPLIFICATION OF THE STRUCTURALLY AND FUNCTIONALLY ALTERED EPIDERMAL GROWTH FACTOR RECEPTOR GENE (c-erb) IN HUMAN BRAIN TUMORS", PAGES 1816-1820, SEE ENTIRE ARTICLE.	1-6,8,9
Y	JOURNAL OF NEURO-ONCOLOGY, VOLUME 7, SUPPL, ISSUED 1989, P.A. HUMPHERY ET AL." AMPLIFICATION AND EXPRESSION OF MUTANT EPIDERMAL GROWTH FACTOR GENES IN HUMAN GLIOMDE", SEE ENTIRE ABSTRACT.	1-6,8,9

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 JULY 1991	13 AUG 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	SUZANNE ZISKA

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	MOLECULAR AND CELLULAR BIOLOGY, VOLUMES, NO.7., ISSUED JULY 1985, G.T. MERLINO ET AL., "STRUCTURE AND LOCALIZATION OF GENES ENCODING ABERRANT AND NORMAL EPIDERMAL GROWTH FACTOR RECEPTOR RNAS FROM A431 HUMAN CARCINOMA CELLS", PAGES 1722-1734, SEE ABSTRACT AND PAGE 1732, PARAGRAPHS 4 AND 6.	1-6,7,8,9
Y	NATURE, VOLUME 309, ISSUED 31 MAY 1984, A. ULLRICH ET AL., "HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR cDNA SEQUENCE AND ABERRANT EXPRESSION OF THE AMPLIFIED GENE IN A431 EPIDERMOID CARCINOMA CELLS", PAGES 418-425, SEE PAGE 418, PARAGRAPH 4; PAGE 422, PARAGRAPH 2; PAGE 423, PARAGRAPH 1; PAGE 424, PARAGRAPH 8.	1-6,8,9
Y	WO,A, 85/03357 (WATERFIELD ET AL.,) 01 AUGUST 1985, SEE ABSTRACT, SEE PAGE 8, LINES 24-25 AND PAGE 9, LINES 1-4.	10

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

GROUP I, CONSISTING OF A FIRST PRODUCT, CLAIMS 1-6,8,9
GROUP II, CONSISTING OF A SECOND PRODUCT, CLAIM 7.
GROUP III, CONSISTING OF A THIRD PRODUCT, CLAIM 10.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.